NF- kappa B Activation Assay Kits

Part Nos. NFKB-1
Storage: Solutions and DTT; -20°C.

Safety Precautions: Although contents are not considered hazardous, avoid skin and eye contact and ingestion. Use gloves and eye protection while using this product.

The instructions below are designated for one 10 cm cell culture dish with cells at 80-90% confluency. Volumes are scalable for additional or other sized cell culture dishes. Scale the reagent volumes used to isolate cytoplasmic and nuclear fractions (i.e. CER-1, NER-1 and DTT) according to the surface area of the culture dish. (For example, for one well in a 6-well dish, scale volumes of CER-1, NER-1 and 1M DTT by 3/10). For multiple dishes or wells, multiply the reagent volumes (for CER-1, NER-1 and IM DTT) by the number of dishes or wells.

Overview: The inhibitory subunit of NF-kappa B, Iκβ, becomes phosphorylated by Iκβ kinase, ubiquitinated and dissociated from the p65(RelA)-p50 complex, which exposes nuclear import signals. The exposure of nuclear import signal targets the NF-kappa B p65(RelA-p50) complex through the nuclear pore into the nucleus, where it binds to regulatory elements and modulates gene transcription.

In the FIVEphoton Biochemicals NF-kappa B Activation Assay Kit NFKB-1, cells are fractionated into cytoplasmic and nuclear fractions, which are then subjected to Western blot analysis. Observing elevated levels of p65 in nuclear fractions relative to other cellular fractions reveals NF-kappa B activation.

Kit Contents

Materials Included With Kit

	Part No.	Description	Volume
1.	CER-1	Cytoplasmic Fractionation Reagent	55 ml
2.	NER-1	Nuclear Fractionation Reagent	3.5 ml
3.	DTT	Dithiothreitol [1]	(solid)

Notes
1. DTT (sufficient to make a 1M solution after addition of 64 μl dH_20).

Materials not provided and required

1. PBS to wash cells.
2. Protease inhibitors, including serine protease Inhibitor.
3. Cell scrapers.
4. Plastic Pasteur pipettes.
5. Refrigerated microcentrifuge for 1.5 ml tubes.
6. ECL Western blot detection kit
7. Antibody to p65 for Western blots
8. Anti-p65 HRP antibody

Protocol: NF-kappa B Activation Assay

Overview: The recommended reagent volumes for the assay to isolated cytoplasmic and nuclear fractions (i.e. CER-1, NER-1, DTT) are detailed below for one 10 cm cell culture dish with 80-90% confluent cells. Adjust reagent volumes accordingly for other sized cell culture dishes based on the surface area of the cell culture dish. For example, for a well in a 6-well dish, use 3/10 the recommended reagent volumes to isolate cytoplasmic and nuclear fractions (i.e. CER-1, NER-1 and 1M DTT). For multiple dishes or wells, multiple the reagent volumes of CER-1, NER-1 and DTT by the number of dishes or wells.

Other plates of cells with parallel treatments can be set aside to obtain total cell lysates using a cell lysis buffer, such as RIPA, for comparison and control to the fractionated materials.

All procedures should be performed rapidly without significant pauses to maintain separation of cytoplasmic and nuclear fractions. Take note that certain substances employed to coat cell culture dishes and enhance adhesion may also activate NF-kappa B and stimulate p65 translocation.

Reagent Preparation

Cytoplasmic Fractionation Reagent (CER-1)	1000 (µl)
Nuclear Fractionation Buffer (NER-1)	60 (µl)
DTT (IM)	1/1000 dilution into CER-1

Reagents for one 10 cm cell culture dish with 80-90% confluent cells.

1. Add 64 µl dH_20 to the provided solid DTT to make a final 1M solution. Vortex until all DTT is suspended.

2. Defrost and prepare 1000 µl Cytoplasmic Fractionation Reagent (CER-1) and 60 µl Nuclear Fractionation Buffer (NER-1) for each 10 cm cell culture dish. Keep both solutions in a tube in ice. Add protease inhibitor cocktail and serine protease inhibitor (such as PMSF) to both the cytoplasmic (CER-1) and nuclear (NER-1) fractionation reagents at concentrations recommended by the manufacturer of the inhibitors immediately prior to use.

3. Add DTT into the Cytoplasmic Fractionation Reagent (CER-1) to make a final concentration of 1 mM DTT immediately prior to use (stock solution is at 1M, therefore dilute DTT 1:1000). Keep solutions ice cold.

Fractionation Steps

1. Remove media and gently wash the cell culture dish twice with 3 ml room temperature PBS pH 7.4. Use suction and a Pasteur pipette to remove as much PBS as possible after washing. Place the cell culture dish on a bed of ice.

2. Cover the cells with 500 µl of ice cold Cytoplasmic Fractionation Reagent (CER-1) with protease inhibitors and DTT. Tilt the cell culture dish several times to cover the dish with a film of Cytoplasmic Fractionation Reagent. Keep the dish on a bed of ice for 5 min.

3. Using a cell scraper, remove cells from the cell culture dish, siphon cells with a wide opening Pasteur pipette, and dispense the cell suspension into a 1.5 ml snap-cap tube. Immediately centrifuge the tube for 3 min at 2500 rpm at 4°C in a refrigerated microcentrifuge.

4. Collect and set aside the supernatant (which contains the **cytoplasmic fraction**) in a tube for storage. Re-suspend the pellet gently in 500 µl of ice cold Cytoplasmic Fractionation Reagent (CER-1) prepared with protease inhibitors and DTT to wash the pellet. You can flick the tube with your fingers several times to re-suspend the pellet. Place the tube in ice for 5 min, and then centrifuge again for 3 min at 2500 rpm, 4° C.

   Remove as much supernatant as possible without disturbing the pellet. *Removal of the supernatant must be performed immediately after centrifugation.* Retain the pellet, which contains the nuclear material. Discard the supernatant.

5. Add 60 µl of ice cold Nuclear Fractionation Reagent (NER-1) with protease inhibitors to the nuclear pellet; include a serine protease inhibitor. Vortex full speed for 1 min and then place the tube in ice for 10 min.

6. Centrifuge at maximum speed (typically 16,000 rpm for a small tube centrifuge) for 10 min. Carefully collect the *supernatant fraction* which contains to the **nuclear fraction.**

   Store the cytoplasmic (from step 4) and nuclear fractions (from step 6) at -80°C. You may wish to aliquot fractionated materials into separate tubes prior to storage at this step.

Resolving Cytoplasmic and Nuclear Fractions in Western Blots

An ECL detection kit (not provided) is required for this step.

Resolving cytoplasmic and nuclear proteins in Western blots. p65 protein may be present at high concentrations in the isolated cytoplasmic and nuclear fractions. To avoid Western blot band saturation. use a 1:1000 dilution of cytoplasmic and nuclear fraction solutions in Laemmli sample buffer as a starting reference dilution prior to loading samples into gels. (Further dilution adjustment may be required to resolve p65 protein in Western blots in a linear range). SDS-PAGE gel lanes can be loaded in the following consecutive order, first for the control, un-stimulated conditions, and a repeat of this same arrangement for samples with the treatment hypothesized to activated NF-kappa B: 1) total cell lysate; 2) cytoplasmic fraction; 3) nuclear fraction.

Western blot development with the provided p65 antibody.

A 10% TRIS-glycine SDS-PAGE gel or a 5-20% gradient TRIS-glycine SDS-PAGE gel are suitable to resolve p65 protein (MW approx. 65kD). Load the gel lanes in the arrangement suggested above.

After the SDS-PAGE gel is resolved, transfer the proteins to a nitrocellulose membrane using standard Western blotting procedures.

Develop the Western blot with antibody to p65 in PBS-T. The recommended incubation times for the p65 antibody with the Western blot membrane are 1 hr at RT, or overnight at 4°C.
(You can also co-develop the Western blot with antibodies to a cytoplasmic protein (e.g. GAPDH) or nuclear protein (e.g. Histone H1) to calibrate the Western blot). After incubation with antibody, wash the membrane three times with PBS-T, with the first wash at 15 min, and the second and third washes at 5 min each.

Afterwards, use an anti-p65 antibody-HRP conjugate in PBS-T. Incubate the HRP antibody with the membrane for 1 hr at RT. Subsequently, wash with PBS-T five times, with the first wash at 15 min, and the subsequent washes 5 minutes each.

Now, use an ECL chemiluminescence Western blot development method to detect p65 in the Western blot membrane.

Data Interpretation: Translocation of p65 into the nucleus can be observed and quantified in Western blots by resolving a total cell lysate, followed by consecutive lanes of the cytoplasmic and nuclear fractions. Use densitometry to measure the Western blot band that corresponds to p65. For each treatment, directly calculate the ratio of p65 protein in the nuclear fraction relative to the cytoplasmic fraction. You can also calibrate data to standard cytoplasmic (e.g.. GAPDH) and nuclear proteins (e.g. histone H1) that are co-detected on the Western blot.

An elevated ratio of p65 protein in the nuclear fraction should be observed with stimuli that activate NF-kappa B.

Troubleshooting

1. Cell fractionation and translocation of p65 were not observed. A potential problem is suspension of the nuclear pellet prior to the removal of the second cytoplasmic fractionation reagent (step 4) due to prolonged and unplanned pauses while performing the isolation. Resolution: Perform procedures as rapidly as possible. Also, make sure protein bands in Western blots are not at saturation levels.

2. The transcription factors in the nuclear fraction resolve poorly in Western blots. Resolution: Dilute samples further in Laemmli sample buffer. Heat sample to 60^{o}C for 5 min and vortex for 1 min instead of boiling in Laemmli sample buffer.

3. Proteolysis of transcription factors was observed in Western blots. Resolution: Add protease inhibitors into CER-1 and NER-1 reagents immediately prior to use.